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Novel serum and bile protein markers predict primary sclerosing cholangitis disease severity and prognosis

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Background & Aims: Prognostic biomarkers are lacking in primary sclerosing cholangitis, hampering patient care and the development of therapy. We aimed to identify novel protein biomarkers of disease severity and prognosis in primary sclerosing cholangitis (PSC).

Methods: Using a bead-based array targeting 63 proteins, we profiled a derivation panel of Norwegian endoscopic retrograde cholangiography bile samples (55 PSC, 20 disease controls) and a Finnish validation panel (34 PSC, 10 disease controls). Selected identified proteins were measured in serum from two Norwegian PSC cohorts (n = 167 [1992–2006] and n = 138 [2008–2012]), inflammatory bowel disease (n = 96) and healthy controls (n = 100).

Results: In the bile derivation panel, the levels of 14 proteins were different between PSC patients and controls (p <0.05); all were confirmed in the validation panel. Twenty-four proteins in the bile derivation panel were significantly (p <0.05) different between PSC patients with mild compared to severe cholangiographic changes (modified Amsterdam criteria); this was replicated for 18 proteins in the validation panel. Interleukin (IL)-8, matrix metallopeptidase (MMP)9/lipocalin (LCN)2-complex, S100A8/9, S100A12 and tryptophan hydroxylase (TPH)2 in the bile were associated with both a PSC diagnosis and grade of cholangiographic changes. Stratifying PSC patients according to tertiles of serum IL-8, but not MMP9/LCN2 and S100A12, provided excellent discrimination for transplant-free survival both in the serum derivation and validation cohort. Furthermore, IL-8 was associated with transplant-free survival in multivariable analyses in both serum panels independently of age and disease duration, indicating an independent influence on PSC progression. However, the Enhanced Liver Fibrosis (ELF®) test and Mayo risk score proved to be stronger predictors of transplant-free survival.

Conclusions: Based on assaying of biliary proteins, we have identified novel biliary and serum biomarkers as indicators of severity and prognosis in PSC.

Lay summary: Prognostic biomarkers are lacking in primary sclerosing cholangitis, hampering patient care and the development of therapy. We have identified inflammatory proteins including calprotectin and IL-8 as important indicators of disease severity and prognosis in bile and serum from patients with primary sclerosing cholangitis.

Keywords: PSC; Biomarker; Prognosis; Calprotectin; IL-8.

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Introduction

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease characterized by progressing inflammation and fibrosis of the intra- and extrahepatic bile ducts, leading to cirrhosis in the majority of patients. PSC is frequently associated with inflammatory bowel disease (IBD) and other immunological diseases. To date, liver transplantation is the only curative treatment in PSC, as there is no medical therapy of proven benefit to halt disease progression. The disease course is highly variable between PSC patients, with transplant-free survival ranging from 12–21 years in different cohorts [1,2].

Predictors of outcome in terms of liver transplantation or death have been proposed, mainly based on biochemical variables (e.g. bilirubin or alkaline phosphatase) or clinical signs (e.g. variceal bleeding or ascites) [3], but currently there are no validated prognostic tools to reliably estimate the prognosis in the individual patient [4,5]. Furthermore, the lack of validated biomarkers reflecting relevant disease inflammatory activity is a major hurdle for clinical trials struggling to demonstrate effect of new therapeutic options. A position paper was recently published by the International PSC Study Group aiming to define surrogate endpoints for clinical trials based on a consensus process [6]. Biomarkers reflecting liver fibrosis including the serum-based Enhanced Liver Fibrosis (ELF®; Siemens Medical Solutions Diagnostics Inc., Tarrytown, NY, USA) test have been reported to predict prognosis in PSC [7]. Although promising biomarkers, a critical question is whether the proposed markers reflect disease activity or disease stage.

In this study, we aimed to explore whether inflammatory biomarkers or biomarkers of fibrosis other than ELF® represent biomarkers of disease activity or disease stage in large-duct PSC. As bile is in immediate contact with the primary target of the disease, i.e. the cholangiocytes (of the bile ducts), we hypothesized that the biliary protein pool would be more likely than serum to accurately reflect disease activity. In order to investigate this we performed an exploratory analysis of a large number of putative biomarker proteins in bile, using a custom multiplex antibody array platform and applying a two-step analysis with validation of significant results in an independent panel. Secondly, we investigated the prognostic potential of key proteins identified in bile, in sera from two independent cohorts by a similar two-step derivation-validation analysis.

Materials and methods

Patient population and data collection

PSC was diagnosed based on typical cholangiographic findings according to acknowledged criteria after the exclusion of secondary causes of sclerosing cholangitis, and excluding small duct PSC [8,9]. The first pathological cholangiography defined the time of PSC diagnosis. For the PSC patients, we queried patient records and research databases for information on clinical and laboratory data, including fever, cholangitis, jaundice, ascites, encephalopathy, esophageal varices, variceal bleeding, IBD status, colorectal or hepatobiliary malignancy, and medication at the time of bile or serum extraction. IBD diagnosis was based on colonoscopy and histology findings. Diagnoses of ulcerative colitis (UC) and Crohn's disease were established by accepted criteria. We retrieved updated information on liver transplantation dates and indications (Table S1) by December 31st 2012 from the Nordic Liver Transplant Registry and data on all-cause death by the same date from the Norwegian Death Registry.

We included two panels of bile samples; an exploratory derivation panel (bile panel-1), and an independent validation panel (bile panel-2) (Fig. S1A). We selected bile samples from PSC patients based on clinical and laboratory data in order to constitute groups of varying disease activity, applying the original Amsterdam score [10] to categorize the PSC patients into groups of "mild" or "advanced" disease (Amsterdam scores 0–2 or $\geq$3, respectively) based on revision of endoscopic retrograde cholangiography (ERC) images. Bile panel-1 consisted of bile samples extracted at ERC from 55 large-duct PSC patients (16 mild, 37 advanced) and 20 disease controls (details in Fig. S1) retrieved from the NoPSC Biobank, Oslo, Norway (Table 1). Bile panel-2 included bile samples extracted at ERC from a Finnish cohort of 34 PSC patients (12 mild, 22 advanced) and 10 controls (Table 1).

For the serum analyses, we adopted a two-step study design including two PSC panels recruited during two different, well-defined time periods (Fig. S1B). The panels were not overlapping. Statistically significant results obtained in serum panel-1 were tested in serum panel-2. Serum panel-1 included serum samples from 167 large-duct PSC patients (median follow-up time of 4 years [range: 0–20.1] from time of serum sampling) collected during 1992–2006. Serum panel-2 included 138 independent PSC patients (median follow-up time of 2.2 years [range: 0.0–4.3] from serum sampling) were collected during 2008–2012 from the NoPSC Biobank. Control serum panels of 100 healthy controls and 96 UC cases from a population based Norwegian cohort were retrieved for comparison [11]. The characteristics of the study populations are shown in Table 2.

Standard biochemical analyses were retrieved from clinical routine laboratory databases including C-reactive protein, white blood count, platelets, creatinine, total bilirubin, albumin, international normalized ratio (INR), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma-glutamyltransferase (GGT). Mayo risk scores were calculated using the algorithm for the revised Mayo risk score [12].

Ethical approval

The protocol was in accordance with Declaration of Helsinki and approved by the regional committee for research ethics in South Eastern Norway (reference number 2011/2572). All study participants provided written, informed consent.

Bile sample preparation

The samples were thawed and 100 µl of bile was added to 150 µl PBS containing 0.1% Tween 20 and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, Missouri, USA), then centrifuged at 4 °C for 10 min at 14,000 rpm. CleanAscite™ (100 µl, Biotech Support Group, NJ, USA) and Protein G Sepharose (50 µl; Sigma-Aldrich) were used for removal of lipids and immunoglobulins, respectively. Proteins were labelled with amine-reactive biotin (NHS-PEO_4-Biotin 1 mg/mL, Thermo Fischer Scientific, IL, USA) for 45 min on ice and separated on a Superdex 200 10/300 GL SEC column coupled to an Äkta Purifier (GE Lifesciences). The mobile phase was PBS containing 0.05% Tween 20 at a flow rate of 0.5 ml/min. After a retention volume of 8 ml, 24 fractions of 0.5 ml each were collected. The fractions were aliquoted and stored at −70 °C until analysis. The samples and fractions were kept cold at all times during the preparation procedure to limit proteolysis.

Antibody array analysis

The production of the bead-based antibody array has been described in detail elsewhere [13]. The antibodies used are commercially available and have been evaluated thoroughly in-house with regard to specificity prior to the current study [14]. A comprehensive list of the antibodies used is provided in Table S2. Antibody-coupled beads were mixed, aliquoted and stored at −70 °C. Mixtures of beads with up to 1728 different color codes were added to SEC fractions containing biotinylated bile proteins. After overnight incubation, the beads were washed three times with PBS containing 1% Tween 20 (PBT). Streptavidin-conjugated phycoerythrin was added to label captured biotinylated proteins (SA-PE, Jackson Immunoresearch). The beads were washed in PBT and analysed with an LSRII flow cytometer (BD Biosciences, San Jose, California, USA) as described previously [13]. Flow cytometry files were analysed with a customized software capable of automatically reading the fluorescent bar codes of microsphere subsets and exporting the median SA-PE fluorescence in text format [15]. The data output is reactivity profiles for all antibodies in the array where molecular size is on the x-axis and fluorescence intensity on the y-axis. Specific target capture is visualized as peaks in the reactivity profile. In order to obtain quantitative data we calculated the peak areas using scripts in Microsoft Excel.

Table 1. Baseline characteristics of PSC patients and controls at the time of bile extraction.

	Bile panel-1		Bile panel-2	
	PSC*	Controls*	PSC	Controls
N	55	20	34	10
Males, n (%)	45 (81.8)	10 (50)	23 (67.6)	NI
Age, median (range)	36.2 (16–70)	45.5 (18–75)	35.4 (19–52)	NI
PSC duration, yr, median (range)	0.2 (0–24)	n.a.	0.0 (0–21)	n.a.
PSC-AIH, n (%)	9 (16.4)	n.a.	1 (2.3)	n.a.
Amsterdam score, median (range)	3.0 (2–4)	n.a.	3.0 (2–4)	n.a.
Amsterdam score ≥3, n (%)	33 (42.9)	n.a.	19 (57.6)	n.a.
IBD, n (%) (final)§	38 (79.2)	NI	25 (78.1)	NI
Ulcerative colitis, n (% of IBD)	27 (71.1)	NI	21 (84.0)	NI
Crohn's disease, n (% of IBD)	6 (15.8)	NI	2 (8.0)	NI
Indeterminate colitis, n (% of IBD)	5 (13.2)	NI	2 (8.0)	NI
Mayo score	−0.04 (−1.96 to −3.28)	n.a.	−0.70 (−1.6 to 2.21)	n.a.
Mayo risk low/intermed./high, n (%)	27/17/8 (51.9/32.7/15.4)	n.a.	24/7/1	n.a.
Treatment				
UDCA, n (%)	21 (38.2)	3 (15)	20 (58.8)	NI
5-ASA, n (%)	18 (32.7)	1 (5)	16 (47.1)	NI
Prednisolone, n (%)	15 (27.3)	2 (10)	1 (2.9)	NI
Laboratory data				
CRP, median (range)	2.9 (0.6–36.9)	3.0 (0.6–38.9)	2.9 (2.9–17.0)	
White blood count, median (range)	6.1 (2.1–18.6)	5.9 (3.3–12.3)	5.4 (3.0–10.0)	
Platelets, 10^9/L, median (range)	270 (70–903)	260 (107–412)	257 (64–475)	
Total bilirubin, μmol/L, median (range)	18.3 (4.8–532)	14.5 (6.0–248.4)	12.0 (4.0–87.0)	
Albumin, g/L, median (range)	41.0 (24.5–49.8)	41.9 (31.2–49.0)	40.1 (24.8–49.6)	
INR, median (range)	1.1 (0.89–2.04)	1.0 (0.9–1.27)	1.0 (0.9–1.7)	
Creatinine, μmol/L, median (range)	64.0 (36.0–89.0)	62.0 (45.0–85.0)	68.0 (44.0–101.0)	
AST, U/L, median (range)	67.0 (16.0–1219.0)	39.0 (16.0–412.0)	41.0 (21.0–342.0)	
ALT, U/L, median (range)	94.0 (14.0–885.0)	51.5 (14.0–613.0)	56.0 (12.0–579.0)	
ALP, U/L, median (range)	226.5 (52.0–788.0)	130.5 (36.0–633.0)	125.0 (47.0–607.0)	
GGT, U/L, median (range)	258.5 (23.0–1620.0)	147.0 (12.0–2334.0)	118.0 (10.0–908.0)	

Patients with large-duct PSC and bile extracted at ERC available were selected retrospectively in two independent cohorts (bile panel-1 and -2) and compared to disease controls with various liver diseases other than PSC.

* Missing data, bile panel-1; PSC: Two patients had missing data for all laboratory data and Mayo score; thus, n = 52 for Mayo score and all laboratory data except INR (n = 46); n = 53 for Amsterdam score. Controls: Two controls have missing data for all lab results; thus, n = 18 for laboratory data except for INR (n = 16) and AST and Mayo score (n = 17).

§ IBD status: Percentages relate to patients with a diagnosis verified by colonoscopy; e.g. in bile panel-1, screening colonoscopy had been performed in 10 out of 16 patients categorized as not having IBD, thus IBD was present in 38/48 = 79.2%. PSC, primary sclerosing cholangitis; AIH, autoimmune hepatitis; IBD, inflammatory bowel disease; n.a., not applicable; CRP, C-reactive protein; INR, international normalized ratio; NS, not significant ($p \geq 0.05$); NI, not investigated; IBD, inflammatory bowel disease; UDCA, ursodeoxycholic acid; 5-ASA, 5-aminosalicylic acid; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyltransferase.

ELISA

Commercial kits from RnD Systems (Minneapolis, MN, US) were used to analyse serum levels of interleukin (IL)-8, S100A12 and the matrix metallopeptidase (MMP)9-lipocalin (LCN)2 complex in frozen serum samples from all 305 PSC patients, the 100 healthy controls and the 96 UC patients. Serum levels of tryptophan hydroxylase 2 (TPH2) were analysed using a commercial kit from Cusabio (Baltimore, MD, US; cat number CSB-EL02410HU) in frozen serum samples from 137 patients in serum panel-2. Inter- and intra-assay coefficients of variation were <10% for the biochemical analyses for all of the associated markers. Plasma levels of calprotectin (S100A8/A9 complex) were analysed in serum panel-2 (n = 138) using a commercial kit (Calprolab, Calpro, Lysaker, Norway).

Statistical analyses

Statistical analyses were performed using SPSS (version 21, SPSS Inc., Chicago, IL, USA), MedCalc (MedCalc Software, Ostend, Belgium) and R (R Foundation for Statistical Computing, Vienna, Austria). *p* values of <0.05 were considered significant. Continuous variables were tested for normal distribution and the Student's *t* test or the Mann-Whitney *U* test was applied as appropriate. Data are presented as median (range). Spearman's rho was used to test for correlations between putative biomarkers and prognostic scores.

Predictive values of putative biomarkers were evaluated by receiver operating characteristic (ROC) curves and are presented as area under the curves (AUCs) with 95% confidence intervals (CI). Optimal cut-off values were determined by ROC analyses according to the Youden's index and we calculated sensitivities and specificities of each protein to differentiate between PSC patients and controls. Primary endpoint was time to death or liver transplantation. We explored the associations between tertiles of IL-8, S100A12, the MMP-LCN2 complex and TPH2 and clinical outcome by the Kaplan-Meier method and tested statistical significance by the log-rank test.

We assessed the associations between clinical, laboratory and radiological features at the time of first diagnosis and clinical outcome by univariate Cox proportional hazards regression analyses. Factors found to be of prognostic significance by univariate analysis were entered into a multivariable model to identify factors independently related to prognosis. From these, a prognostic model was constructed. IL-8 and ELF® and Mayo score were assessed in different Cox models owing to collinearity (r >0.5). Lasso analysis [16] using the R penalized package was performed to find subsets of biomarkers best discriminating between PSC *vs.* controls and severe *vs.* mild PSC, respectively. To avoid overfitting due to multiple explanatory variables (the 63 biomarkers), we first trained the lasso models on bile panel-1, using cross-validation to find the optimal penalization parameter. The coefficient estimates of these trained models were used to evaluate model performance (AUC) using bile panel-2 as an independent test data set. In all tests, *p* values of <0.05 were considered statistically significant.

Table 2. Baseline characteristics of the PSC patients in serum panel-1 and -2.

	Derivation panel			Validation panel		
	Tx-free survivors[A]	Tx/death[B]	*p* value	Tx-free survivors[C]	Tx/death[D]	*p* value
N	52	115		91	47	
Males, n (%)	38 (73.1)	86 (74.8)	n.s.	73 (80.2)	34 (72.3)	n.s.
Age, mean (95% CI)	35.1 (31.4, 38.8)	42.2 (39.8, 44.6)	0.002	38.1 (35.4, 40.9)	47.7 (44.1, 51.2)	<0.001
Age at diagnosis, median (range)	32.2 (13.8–70.1)	35.1 (13.2–70.0)	n.s.	31.0 (14.5–65.8)	42.9 (20.7–71.5)	0.001
PSC duration, years, median (range)	0.2 (0–20.9)	2.9 (−0.2–22.4)	<0.001	0.6 (−0.6–29.0)	3.2 (0–26.5)	0.039
IBD ever, n (%)*	41 (87.2)	98 (89.9)	n.s.	70 (87.8)	32 (68.1)	n.s.
Time of follow-up, yr, median (range)	11.8 (7.2–20.1)	1.4 (0–17.5)	<0.001	2.8 (0.53–4.29)	0.4 (0–3.9)	<0.001
Liver transplant, n (%)	0	85 (73.9)		0	33 (70.2)	
Death as endpoint, n (%)	0	30 (26.1)		0	14 (29.8)	
Mayo risk score, median (range)*	−0.17 (−2.24–2.91)	1.44 (−1.39–5.26)	<0.001	−0.24 (−2.37–3.20)	1.40 (−0.95–4.13)	<0.001
Laboratory data						
ALP, U/L, median (range)*	413 (124–1883)	741 (70–3100)	0.022	204 (51–1459)	314 (82–892)	<0.001
AST, U/L, median (range)*	57 (8–556)	108 (10–1012)	0.009	56 (16–1219)	100 (32–585)	<0.001
ALT, U/L, median (range)*	110 (9–780)	108.5 (8–524)	n.s.	74 (14–885)	99 (22–768)	n.s.
Albumin, g/L, median (range)*	40 (22–50)	34 (15–46)	<0.001	42 (28–50)	37 (23–46)	<0.001
Total bilirubin, μmol/L, median (range)*	16 (5–227)	54 (5–567)	<0.001	13 (3–175)	46 (5–532)	<0.001
Creatinine, μmol/L, median (range)*	73.5 (51–98)	73 (41–216)	n.s.	67 (39–91)	61 (37–111)	n.s.
Platelets, 10^9/L, median (range)*	256 (10–756)	223 (35–879)	0.008	290 (65–903)	277 (22–759)	n.s.

Differences between Tx-free survivors and patients who died or underwent liver transplantation were tested using Student's *t* test or the Mann-Whitney *U* test for normally and non normally distributed data, respectively.
Serum panel-1 and -2 represent independent PSC panels.
Tx, liver transplantation; n.s., not significant ($p \geq 0.05$); IBD, inflammatory bowel disease; ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase.
* Refers to a subpopulation of (A) n = 44–49, (B) n = 81–109, (C) n = 83–90, (D) n = 45–47.

Results

Explorative analysis of putative biomarkers by protein array analysis

The antibody array consisted of 144 well-characterized antibodies targeting 63 proteins described to be involved in pathways related to inflammation and fibrosis. Antibody array analysis revealed 14 proteins that were significantly different between PSC patients and controls (Table S3). The PSC patients were classified as having either early or advanced disease (ERC-based Amsterdam score 0–2 or ≥3, respectively). Comparing early and advanced PSC, 24 proteins showed significant differences (Table S4). Subsequent analysis in an independent bile panel-2 (Table 1) replicated significant results for 13 proteins differing between PSC and controls (Table S3). Eighteen of the 24 proteins differing between mild and advanced disease in bile panel-1 were also significantly different in bile panel-2 (Table S4).

Evaluation of the diagnostic utility of the novel bile biomarkers in PSC

All of the 13 proteins that were consistently showing a significant difference between PSC patients and controls were evaluated by ROC-AUC analyses in bile panel-1 and bile panel-2 (Table 3). The proteins performing best as single discriminators in the two panels combined were IL-8, S100A12, LCN2, S100A8 (calprotectin) and ITGB2. However, lasso analysis (optimizing subset selection in order to enhance the prediction accuracy of the model it produces) identified ICAM1, MMP7, S100A4 and S100A12 as the group of markers that collectively best distinguished PSC from controls (Fig. 1A).

Evaluation of the utility of the novel bile biomarkers in measuring disease severity in PSC

In order to evaluate the ability of putative biomarkers to distinguish between mild and advanced disease, all proteins consistently showing significant differences between mild and advanced PSC in both bile panels, were evaluated by ROC-AUC analyses in bile panel-1 and bile panel-2, respectively (Table 4). The top three single targets best fit to distinguish mild from advanced PSC were C5, calprotectin and TIMP1 (bile panel-1) and S100A12, MPO, calprotectin and S100A7 (bile panel-2). Combining ROC-AUC results, the targets best fit to distinguish advanced from mild PSC were calprotectin (mean AUC 0.800), S100A12 (mean AUC 0.787), TGFB1 (mean AUC 0.786) and LCN2 (mean AUC 0.783). Lasso analysis identified calprotectin alone as the protein best capturing the difference between mild and advanced PSC (Fig. 1B).

Evaluation of the predictive value of the putative prognostic biomarkers in peripheral blood

Serum analyses were performed in two independent cohorts described in Table 2. In bile, seven proteins were significantly different between both PSC patients and controls and between PSC patients with mild and advanced disease (IL-8, MMP9/LCN2 complex, S100A12, TPH2, ITGB2 and calprotectin), and we considered these as good candidates for further investigation.

In serum, IL-8 levels were significantly higher in PSC compared to UC patients and healthy controls (median [range]: 39 [1–1891] pg/ml, 10.4 [1–1150] pg/ml and 3 [10–229] pg/ml, respectively; $p < 0.001$ for both comparisons; Fig. 2A). PSC patients had higher levels of IL-8 compared to subgroups of UC

Table 3. Evaluation of putative biomarkers for PSC detection by ROC-AUC analyses.

	Bile panel-1						Bile panel-2					
Target	**Cut-off**	**AUC**	**95% CI**	***p* value**	**Sensitivity**	**Specificity**	**Cut-off**	**AUC**	**95% CI**	***p* value**	**Sensitivity**	**Specificity**
CD14	382	0.675	0.557, 0.779	0.02	94.6	40.0	1062	0.821	0.674, 0.921	<0.001	72.7	90.0
CLU	6606	0.734	0.619, 0.829	<0.001	69.1	75.0	3779	0.827	0.681, 0.925	<0.001	66.7	90.0
CXCL10	1365	0.686	0.569, 0.789	0.01	74.6	65.0	955	0.852	0.710, 0,941	<0.001	87.9	80.0
IL-8	12,115	0.734	0.619, 0.829	<0.001	72.7	70.0	5710	0.897	0.766, 0.969	<0.001	72.7	100.0
ITGB2	1665	0.692	0.575, 0.793	0.006	74.6	65.0	1907	0.924	0.801, 0.983	<0.001	84.9	100.0
LCN2	46,435	0.699	0.582, 0.800	0.007	70.9	70.0	40,193	0.945	0.830, 0.992	<0.001	81.8	100.0
LDHA	3461	0.660	0.541, 0.761	0.03	70.9	65.0	544	0.830	0.685, 0.927	<0.001	66.7	100.0
MMP7	25,117	0.717	0.601, 0.815	0.002	72.7	70.0	28,837	0.824	0.678, 0.923	<0.001	57.6	100.0
MMP9	4355	0.725	0.609, 0.821	0.004	89.1	60.0	6024	0.882	0.747, 0.960	<0.001	81.8	100.0
S100A4	22,260	0.730	0.615, 0.826	<0.001	67.3	85.0	18,437	0.879	0.743, 0.958	<0.001	72.7	100.0
S100A8	82,136	0.749	0.636, 0.842	<0.001	76.4	70.0	78,482	0.861	0.721, 0.947	<0.001	72.7	90.0
S100A12	13,512	0.764	0.651, 0.854	<0.001	85.5	65.0	32,916	0.876	0.739, 0.956	<0.001	72.7	100.0
TPH2	1040	0.679	0.561, 0.782	0.01	83.6	50.0	1901	0.891	0.758, 0.965	<0.001	78.8	100.0

The statistical analysis used is ROC-AUC analysis, as stated in the text.
ROC-AUC analysis was performed for each protein target showing significantly different levels in bile from primary sclerosing cholangitis (PSC) patients and disease controls in both bile panel-1 and bile panel-2. The optimal cut-off was determined by Youden's index.
AUC, area under the curve; CI, confidence interval; ROC-AUC, area under the curve of the receiver operating characteristics curve.

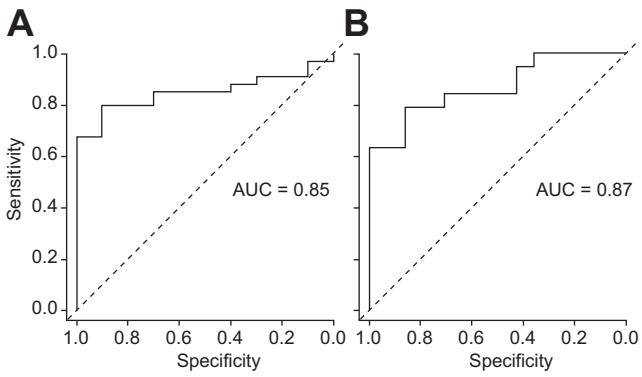

Fig. 1. Identification of biomarker panels by lasso analysis. Lasso analysis identified (A) ICAM1, MMP7, S100A4 and S100A12 as the group of markers that collectively best distinguished primary sclerosing cholangitis (PSC) from controls (AUC 0.85), and (B) S100A8 (S100A8/A9 complex; calprotectin) as the single marker best distinguishing mild from advanced PSC, with no added value by addition of other markers.

patients either active or in remission (median [range] 12 [1–1038] pg/ml, p <0.001, and 8 [1–28] pg/ml, p <0.001, respectively). IL-8 correlated with the ELF® score and Mayo score (serum panel-1: Spearman's rho 0.68 and 0.59, respectively; p <0.001 for both; serum panel-2: Spearman's rho 0.64 and 0.46, respectively; p <0.001 for both). Exploration of serum panel-1 by Kaplan-Meier plot analysis showed a significant inverse association between IL-8 levels and transplant-free survival for PSC patients (log-rank test: Chi-square 31.2, p <0.001; Fig. 2B). This was replicated in serum panel-2 (Fig. 2C).

PSC patients had lower serum levels of the MMP9/LCN2 complex compared to either UC or healthy controls (median [range] 3.9 [0.2–150.0], 21.6 [0.9–130.0] and 15.3 [1.4–65.6], respectively; p <0.001 for both comparisons). Likewise, S100A12 levels were lower in PSC compared to either UC or healthy controls (median [range] 7.4 [1.0–30.0], 8.6 [1.0–28.2] and 9.2 [1.0–16.9], respectively; p = 0.001 and p <0.001, respectively). No associations with survival were found for S100A12, the MMP9-LCN2 complex or TPH2 (log-rank test: Chi-square 0.73 [p = 0.70], 3.60 [p = 0.17] and 1.63 [p = 0.20], respectively; Fig. S2A and B).

Plasma (only available from panel-2) calprotectin (S100A8/A9) levels were not significantly different between PSC patients and controls (median [range] 414.6 [89.2–2501.0] and 424.7 [153.9–1557.2], respectively; p = 0.62; Fig. S2C). No significant differences in survival were found for tertiles of calprotectin using log-rank test (p = 0.32, Fig. S2D), although further exploration of the data identified a weak association with poorer prognosis in the upper quartile when comparing with the other patients (p = 0.03; Fig. S2E and F).

Prognostic modeling of novel and previously reported biomarkers in PSC

Results from univariate Cox proportional hazards regression analysis including all patients with complete data in the derivation and the validation panels, respectively, are listed in Table 5. Age, age at diagnosis, bilirubin, albumin, AST, INR, the Mayo risk score, ELF score and IL-8 showed a significant (p <0.05) association with transplant-free survival consistently in both panels. We have previously reported that the ELF score and Mayo risk score are both independently associated with transplant-free survival [7]. Owing to collinearity (rho >0.5), we assessed IL-8 in a Cox model separate from ELF and Mayo score in the multivariable analyses. Other variables showing collinearity with IL-8 were also excluded.

In serum panel-1, multivariable Cox regression analysis including IL-8 and sex, age at diagnosis, PSC duration, serum platelets and albumin, yielded a final model including IL-8 (HR 1.18 95% CI [1.01, 1.37], p = 0.04), age at diagnosis (HR 1.02, 95% CI [1.00, 1.04], p = 0.02), PSC duration (HR 1.09, 95% CI [1.05, 1.14], p <0.001) and albumin (HR 0.90, 95% CI [0.87, 0.93], p <0.001); whereas in serum panel-2, the identical analysis yielded a final model including IL-8 (HR 1.50, 95% CI [1.18, 1.91], p = 0.001), age at diagnosis (HR 1.05, 95% CI [1.02, 1.07], p <0.001), platelets (HR 0.59, 95% CI [0.35, 0.99], p = 0.04) and albumin (HR 0.92, 95% CI [0.87, 0.97], p = 0.002) (Table 6). When patients in serum panel-1 were divided by the median Mayo score, repeating the analysis showed a final model including IL-8 in the low-Mayo score group but not in the high-Mayo score group (Table S5). In a multivariable analysis adding serum

Table 4. Evaluation of the usefulness of putative biomarkers in bile to distinguish mild and advanced PSC.

	Bile panel-1						Bile panel-2					
Target	**Cut-off**	**AUC**	**95% CI**	***p* value**	**Sensitivity**	**Specificity**	**Cut-off**	**AUC**	**95% CI**	***p* value**	**Sensitivity**	**Specificity**
C5	1358	0.745	0.606, 0.855	0.002	87.9	60.0	3188	0.713	0.526, 0.858	0.021	57.9	84.6
CAT	31,887	0.685	0.543, 0.806	0.023	75.8	65.0	58,681	0.810	0.632, 0.926	<0.001	63.2	100.0
CD177	1898	0.717	0.576, 0.832	0.004	72.7	75.0	5364	0.717	0.530, 0.861	0.021	57.9	100.0
CHI3L1	1078	0.675	0.532, 0.797	0.022	63.6	80.0	2031	0.729	0.543, 0.870	0.013	42.1	100.0
IL-1B	371	0.680	0.538, 0.802	0.026	72.7	65.0	522	0.783	0.603, 0.909	<0.001	57.9	100.0
IL-6	359	0.701	0.559, 0.819	0.008	69.7	70.0	1484	0.688	0.501, 0.839	0.048	42.1	92.3
IL-8	49,225	0.702	0.560, 0.819	0.008	57.6	85.0	23,956	0.854	0.685, 0.953	<0.001	80.0	84.6
ITGB2	5049	0.680	0.537, 0.801	0.020	60.6	80.0	5056	0.842	0.670, 0.946	<0.001	73.7	92.3
LCN2	74,071	0.720	0.579, 0.834	0.003	72.7	75.0	49,516	0.846	0.675, 0.949	<0.001	89.5	69.2
MMP8	22,819	0.670	0.527, 0.793	0.028	54.6	85.0	19,839	0.830	0.656, 0.939	<0.001	63.2	92.3
MMP9	114,780	0.699	0.558, 0.818	0.007	48.5	95.0	29,733	0.794	0.614, 0.916	<0.001	63.2	92.3
MPO	37,498	0.680	0.538, 0.802	0.025	66.7	70.0	17,003	0.866	0.699, 0.960	<0.001	89.5	76.9
S100A7	2259	0.692	0.551, 0.812	0.011	54.6	90.0	2109	0.858	0.690, 0.956	<0.001	79.0	84.6
S100A8	261,601	0.742	0.604, 0.853	<0.001	66.7	80.0	206,305	0.858	0.690, 0.956	<0.001	79.0	84.6
S100A12	408,500	0.682	0.540, 0.803	0.016	51.5	85.0	111,312	0.891	0.730, 0.973	<0.001	79.0	92.3
TGFB1	3943	0.717	0.576, 0.832	0.004	66.7	75.0	4917	0.854	0.685, 0.953	<0.001	63.2	100.0
TIMP1	221	0.728	0.588, 0.841	0.001	63.6	85.0	221	0.769	0.587, 0.899	0.002	63.2	92.3
TPH2	3181	0.683	0.541, 0.804	0.016	45.5	90.0	5118	0.733	0.547, 0.873	0.009	47.4	100.0

The statistical analysis used is ROC-AUC analysis, as stated in the text.
ROC-AUC analysis was performed for each protein target showing significantly different levels in bile from primary sclerosing cholangitis (PSC) patients with mild and advanced disease as assessed by the Amsterdam score in both bile panel-1 (N = 53) and bile panel-2 (N = 32). The optimal cut-off was determined by Youden's index.
AUC, area under the curve; CI, confidence interval; ROC-AUC, area under curve of the receiver operating characteristic.

bilirubin to the analysis, IL-8 was still an independent predictor of transplant-free survival in serum panel-2; whereas in serum panel-1, IL-8 was not part of the final model (Table S6). The Mayo score and ELF score were assessed in separate analyses excluding IL-8, and the final models included independent associations of Mayo score and ELF score with outcome as previously reported (data not shown) [7]. Application of an alternative endpoint defined as liver failure related transplantation or death, confirmed an independent association of IL-8 with prognosis (Fig. S3 and Table S7).

Given the possible association between plasma calprotectin and progression only investigated in panel-2, we also performed univariable Cox regression with Ln calprotectin, which was significant (HR 1.60 [1.04, 2.47], $p = 0.03$). In multivariable models, plasma calprotectin was an independent predictor in all models (Table S8), with a final model including Ln calprotectin (HR 1.65 [1.07, 2.54], $p = 0.03$), Mayo score (HR 1.54 [1.15, 2.08], $p = 0.004$) and ELF test (HR 1.41 [1.09, 1.83], $p = 0.008$).

Discussion

In this exploratory study, through the use of advanced antibody array technology we identified in ductal bile a number of proteins related to inflammation and fibrosis that were associated with disease activity or severity in a large number of PSC patients. We considered as true findings only results that were validated by replication in an independent panel. Out of the eighteen proteins differing between mild and advanced PSC, lasso analysis showed that calprotectin alone best captured the difference between mild and severe disease in PSC. Serum analyses identified IL-8 as the best predictor of transplant-free survival in PSC out of the identified inflammatory markers.

In the present study we found a widely different proteomic profile between patients with and without PSC, and between PSC patients with early and advanced disease. In contrast to previous reports focusing on the predictive value of markers of fibrosis like ELF in PSC, the results of the present study highlight the importance of inflammation. From the lists of suggested diagnostic or prognostic markers, seven proteins were associated both with PSC as such, and with PSC severity, namely IL-8, ITGB2, LCN2, MMP9, calprotectin, S100A12 and TPH2. Interestingly, all of these are markers of inflammation, except for TPH2 which is a marker of proliferation [17].

IL-8 attracts and activates neutrophils, leading to the release of a wide variety of substances including S100A8/A9, S100A12 and MMP9, also highlighted by the present data. Previous reports have also demonstrated elevated serum IL-8 levels in various chronic liver diseases, with increased levels associated with advanced stages of fibrosis and cirrhosis. Interestingly, higher levels were found in cholestatic liver disease compared to other etiologies, and in PSC patients compared to healthy controls [18,19]. IL-8 has also been implicated in biliary atresia, another cholestatic disease [20]. Moreover, an elevated expression of IL-8 was observed in cholangiocytes from PSC livers compared to cholangiocytes from the livers of patients with PBC, hepatitis C virus and normal liver [21]. In the same study, exposure to LPS induced a state of hypersecretion of IL-8 by the biliary epithelial cells, suggesting a possible link to IBD and the leaky gut hypothesis. Overall, the very high concentrations of IL-8 both in bile and serum and the link to disease severity as well as prognosis suggest that it has an important role in PSC, and that the cellular source, at least in part, may be the biliary epithelium. Of note, cholangiocyte senescence with transition of cholangiocytes to a pathological state involving, amongst other things, hypersecretion of IL-8, has been suggested as a putative contributing factor in PSC pathogenesis [21]. Studies to further detail the roles of IL-8 in cholangiocyte biology and its relevance in biliary inflammation appear warranted based on the available data.

Biliary calprotectin was both associated with PSC *per se* and PSC severity, as evaluated by the Amsterdam score. This is in line with previous reports proposing calprotectin as a bile protein

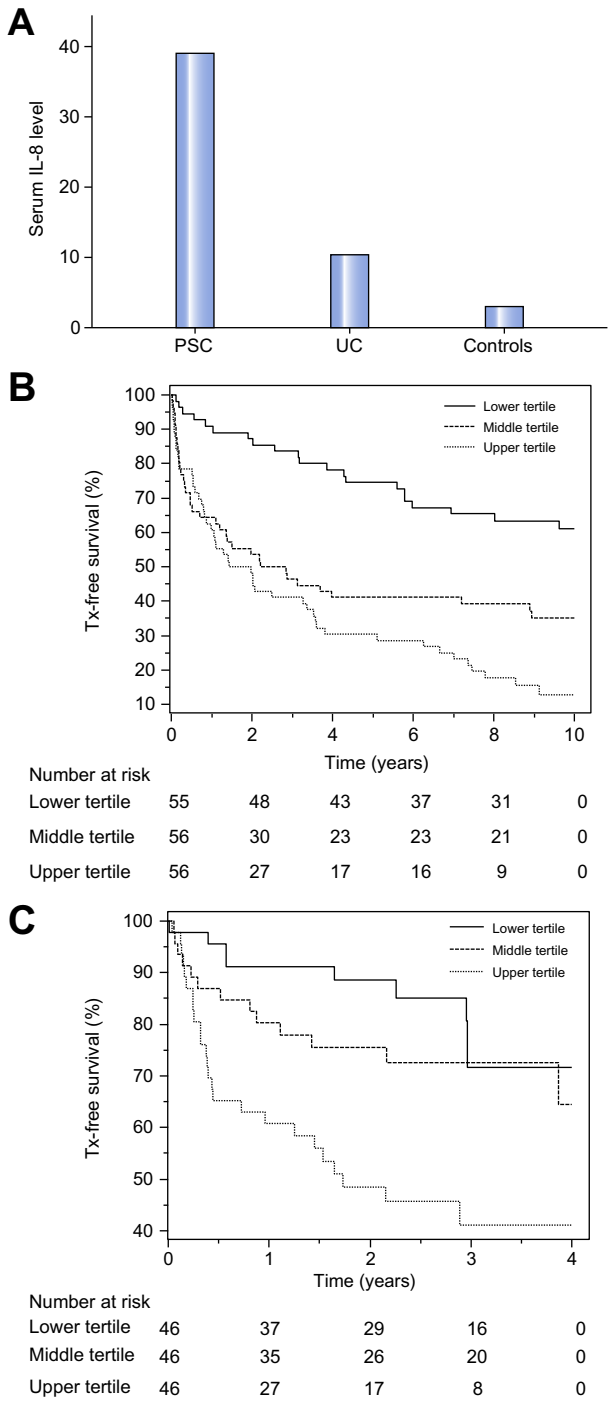

Fig. 2. Prediction of transplant-free survival by serum biomarkers (IL-8). (A) Elevated serum IL-8 levels in PSC (n = 305) compared to UC (n = 98) or healthy controls (n = 100). (B and C) Kaplan-Meier curves of time to transplantation (Tx) or death for PSC patients stratified into tertiles of IL-8 for serum panel-1 (B) and serum panel-2 (C), respectively, illustrating that in patients in the lower tertile of IL-8 (solid line), survival was significantly longer compared to the intermediate and high IL-8 tertiles (dashed line and dotted line, respectively). PSC, primary sclerosing cholangitis; UC, ulcerative colitis.

marker for disease activity in PSC [22–24]. Calprotectin was previously shown to correlate with the Mayo risk score in PSC patients regardless of the presence or absence of IBD. Calprotectin is involved in the promotion of inflammatory responses in several autoimmune diseases as well as in inflammation-associated cancer, acting in part via Toll-like receptor 4, which has also been implicated in PSC [22]. In IBD, calprotectin is a well-established fecal marker of colonic inflammation and a commercially available test is widely used in the clinical follow-up [25]. In analogy to this, it appears an intriguing opportunity whether assays for calprotectin could be modified for the assessment of calprotectin levels in bile sampled at ERC to provide severity assessments. The observed association between plasma calprotectin and disease progression in one study panel is promising but needs validation in independent cohorts.

We have previously demonstrated that a biomarker panel for fibrosis, the ELF score, based on three direct components of liver fibrogenesis, is well suited to stratify PSC patients into groups of differing prognosis [3]. In the present study, we show that the Mayo risk score and ELF score are likely to be stronger predictors of clinical outcome compared to IL-8. However, IL-8 was associated with transplant-free survival in multivariable analyses in two independent PSC cohorts, indicating an important influence on PSC progression. Our findings underline the implication of IL-8, as a marker of inflammation, in the pathogenesis of PSC. Interestingly, novel data confirming increased levels of IL-8 in the bile of PSC patients compared to controls indicate that IL-8 stimulates proliferation and fibrogenic gene expression in primary human cholangiocytes, linking the primarily inflammatory marker IL-8 also to fibrosis development [26]. Taken together, the inflammatory pathways represented by IL-8 may thus harbor potential treatment targets for PSC, and mechanistic studies to elaborate the relationship between IL-8 production and fibrosis development are warranted.

The large number of putative biomarkers explored using extensively validated antibodies and the validation of results in independent patient panels represent strengths of the present study. Limitations include its retrospective design. In future, improved proteomic techniques might allow a more comprehensive unbiased analysis in bile. Any bias of ERC indication (diagnostic *vs.* intervention) was not evaluated due to uncertainty of available retrospective data and should be assessed in subsequent prospective cohorts. The bile panel disease control groups included non-cholestatic diseases perhaps more relevant for investigation of pathogenetic pathways than assessment of diagnostic utility; however, ERC-bile samples were scarce, complicating tailoring of optimal control panels. All patients in the present series were included at a tertiary referral center and may represent more severely affected subgroups. The long follow-up allowed us to use all-cause death and liver transplantation as combined endpoints, however, we acknowledge that lead-time bias may influence the accuracy of such measures.

Through exploration of a wide range of putative biomarkers in the bile of PSC patients in two independent patient panels, we have identified multiple proteins related to inflammation distinguishing mild from advanced disease, including IL-8 and calprotectin. Serum analyses in two independent PSC patient panels demonstrated that serum IL-8 was predictive of liver transplantation free survival, suggesting a contribution of IL-8 associated inflammatory mechanisms to PSC pathogenesis. Subanalyses in groups defined by Mayo score seemed to support a role of IL-8 particularly in early PSC. While markers of disease stage (Mayo risk score) and fibrosis (ELF score) were stronger predictors of survival than IL8, our data strongly suggest that inflammatory

Table 5. Univariate Cox regression analyses of factors affecting the transplant-free survival in PSC patients in serum panel-1 and -2, respectively.

	Serum panel-1				Serum panel-2			
	HR	95% CI	*p* value	N	HR	95% CI	*p* value	N
Age	1.03	1.02, 1.04	<0.001	167	1.05	1.02, 1.07	<0.001	138
Sex	1.10	0.71, 1.70	0.683	167	0.62	0.33, 1.19	0.151	138
Age at diagnosis	1.02	1.01, 1.03	0.005	167	1.04	1.02, 1.06	<0.001	138
PSC duration	1.07	1.04, 1.10	<0.001	167	1.03	0.99, 1.07	0.211	138
IBD status ever	0.70	0.35, 1.39	0.308	156	1.57	0.85, 2.89	0.152	137
Bilirubin	1.80	1.52, 2.12	<0.001	153	2.24	1.73, 2.88	<0.001	130
Albumin	0.89	0.86, 0.91	<0.001	141	0.86	0.82, 0.91	<0.001	129
ALP	1.23	0.93, 1.63	0.141	139	2.35	1.57, 3.53	<0.001	130
AST	1.33	1.05, 1.68	0.017	153	1.73	1.28, 2.35	<0.001	130
ALT	0.90	0.74, 1.10	0.307	154	1.17	0.84, 1.63	0.361	130
Platelet count	0.68	0.52, 0.89	0.005	125	0.66	0.43, 1.01	0.053	129
APRI score (AST/TRC)	0.99	0.94, 1.04	0.690	121	1.38	1.17, 1.63	<0.001	129
INR	3.43	1.92, 6.12	<0.001	95	17.98	5.01, 64.52	<0.001	113
Variceal bleeding	0.54	0.26, 1.11	0.093	147	2.59	0.80, 8.35	0.112	138
Mayo score	1.75	1.52, 2.02	<0.001	125	2.06	1.67, 2.54	<0.001	129
ELF score	2.27	1.89, 2.73	<0.001	167	2.29	1.78, 2.95	<0.001	138
MMP9-LCN2	0.99	0.95, 1.03	0.541	167	0.99	0.98, 1.01	0.482	138
S100A12	1.02	0.98, 1.07	0.339	167	0.94	0.86, 1.02	0.148	138
TPH2	n.a.	n.a.	n.a.	n.a.	1.00	1.00, 1.00	0.102	138
S100A8/A9	n.a.	n.a.	n.a.	n.a.	1.60	1.04, 2.47	0.033	138
IL-8	1.00	1.00, 1.00	0.003	167	1.00	1.00, 1.01	0.001	138

The table shows the results of univariate Cox regression analyses of relevant clinical and laboratory variables.
Bilirubin, ALP, AST, ALT, and platelet count were transformed by the natural logarithm prior to regression analyses due to a right-skewed distribution.
ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CI, confidence interval; ELF, enhanced liver fibrosis; HR, hazard ratio; INR, international normalized ratio; IBD, inflammatory bowel disease; IL8, interleukin-8; MMP9-LCN2, the complex of matrix metalloproteinase-9 and lipocalin-2; PSC, primary sclerosing cholangitis; S100A8/A9, calprotectin; S100A12, S100 calcium-binding protein A12 (calgranulin-C); TPH2, tryptophan hydroxylase 2.

Table 6. Multiple Cox proportional hazards model.

	Serum panel-1			Serum panel-2		
	HR	95% CI	*p* value	HR	95% CI	*p* value
Age at diagnosis	1.02	1.00, 1.04	0.02	1.05	1.02, 1.07	<0.001
PSC duration	1.09	1.05, 1.14	<0.001			
Thrombocytes				0.59	0.35, 0.99	0.04
Albumin	0.90	0.87, 0.93	<0.001	0.92	0.87, 0.97	0.002
IL-8	1.18	1.01, 1.37	0.04	1.50	1.18, 1.91	0.001

The table shows the results of multivariable Cox regression analysis.
The analysis was performed in serum panel-1 (n = 116) and in serum panel-2 (n = 128), respectively, entering the following variables: Sex, age at diagnosis, PSC duration, serum albumin, serum platelets and serum IL-8. The natural logarithm was used for platelets and IL-8 because of right-skewed distribution. Sex and all variables showing significant association in the univariate analyses were included, except for variables showing collinearity with IL-8 (r >0.5) in any of the panels (ELF score, Mayo score, serum bilirubin and serum AST) which were assessed in separate analyses, see Table S4). Age and age at diagnosis were strongly correlated, and therefore only age at diagnosis was included (similar results were achieved if age was substituted for age at diagnosis).

markers should be included alongside fibrosis markers in the further search for parameters providing a comprehensive description of PSC severity and progression.

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Conflict of interest

Dr. Dale reports personal fees from CALPRO AS, outside the submitted work; In addition, Dr. Dale is a patent Inventor and patent holder for the calprotectin analysis licensed. All the other authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript. Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

MV contributed to the designing of the study, data analysis and the writing and revision of the paper; AH contributed to the designing of the study, laboratory analysis and the writing and revision of the paper; JRH contributed to the designing of the study, data analysis and the writing and revision of the paper; SN contributed to the planning and performance of the statistical analyses, and revision of the manuscript; ES contributed to the designing of the study and revision of the paper; EM, ID and LWT contributed to the laboratory analyses and the revision of

the paper; VP, KL, BM and MVa contributed to the sample acquisition and the revision of the paper; SJLBZ, FGS, OHG and PLMJ contributed to the data interpretation and revision of the paper; TU contributed to the laboratory analyses and revision of the paper; HR contributed to the designing of the study, the statistical analyses, and the writing and revision of the paper; CYP contributed to the designing of the study, the cholangiographic grading, and revision of the paper; KMB contributed to the designing of the study, data collection and revision of the paper; MF contributed to the designing of the study, the acquisition of bile samples and clinical data, and revision of the paper; THK and FLJ contributed to the conception and designing of the study and the writing and revision of the paper.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2017.01.019.

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